RayBio® Cell-Based
Human/Mouse/Rat EGFR (Multi-site) Phosphorylation ELISA Sampler Kit

For the semi-quantitative detection of phosphorylated human, mouse, or rat EGFR (Tyr845), (Tyr992), (Tyr1068), (Activated) and total EGFR in adherent whole cell lines.

User Manual
(Revised July 16, 2015)

Cat#: CBEL-EGFR-SK (2 plate kit)

Please read manual carefully
before starting experiment

Tel: (Toll Free) 1-888-494-8555 or +1-770-729-2992; Fax: +1-770-206-2393;
Website: www.raybiotech.com Email: info@raybiotech.com

Cell-Based Human/Mouse/Rat EGFR (Multi-Site) Phosphorylation ELISA Sampler Kit

TABLE OF CONTENTS

I. Introduction 2
II. How It Works 3
III. Reagents and Storage 4
IV. Additional Reagents Required 4
V. Reagent Preparation 5
VI. Assay Procedure 6
VII. Assay Procedure Summary 9
VIII. Quality Control Data 10
IX. References 13
X. Troubleshooting Guide 14

I. INTRODUCTION

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the living cells including proliferation, differentiation and metabolism. A large number of protein kinases and phosphatases have been extensively investigated, and have been shown to be involved in signal transduction pathways.

The RayBio® Cell-Based EGFR ELISA Sampler Kit is a very rapid, convenient and sensitive assay kit which can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of EGFR (Tyr845), EGFR (Tyr992), EGFR (Tyr1068) and EGFR (Activated) phosphorylation and screening the effect of various treatment, inhibitors (such as siRNA or chemicals), or activators in cultured human, mouse and rat cell lines. **EGFR (Tyr1068) and EGFR (Activated) antibodies only can detect human cell lines**. By determining specific protein phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in preparing cell lysate and performing an analysis of Western Blot. In the Cell-Based EGFR ELISA Sampler Kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatment, inhibitors or activators. After blocking, anti-phospho-protein specific antibody or anti-pan-protein specific antibody (primary antibody) is pipetted into the wells and incubated. The wells are washed, HRP-conjugated anti-rabbit or mouse IgG (secondary antibody) is added to the wells. The wells are washed again, a TMB substrate solution is added to the wells and color develops in proportion to the amount of protein. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

See Figure 1 below for an illustration.

II. HOW IT WORKS

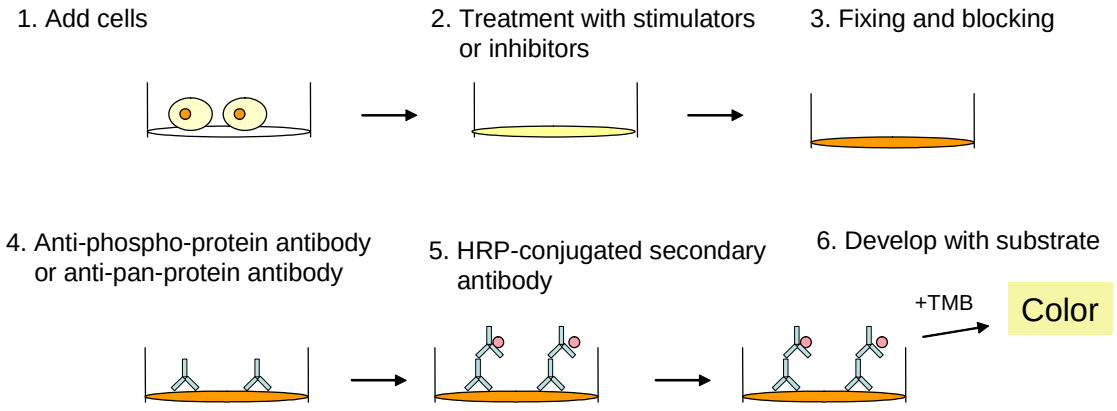

Fig.1. Cell-Based protein phosphorylation procedure

III. REAGENTS AND STORAGE

Store entire kit at ≤ -20 °C immediately upon arrival. Kit must be used within

ITEM	COMPONENT	2 PLATE KIT	STORAGE AFTER INITIAL THAW*
A	Uncoated 96-Well Microplate	2 plates	Room Temperature
B	20X Wash Buffer A Concentrate	1 vial (30 ml)	2-8 °C
C	20X Wash Buffer B Concentrate	1 vial (30 ml)	
D	Fixing Solution	1 vial (30 ml)	
E	30X Quenching Buffer Concentrate	1 vial (2 ml)	
F	5X Blocking Buffer Concentrate	1 vial (20 ml)	2-8 °C (1 month)
G-1	20X Mouse Anti-phospho (Tyr845) EGFR Concentrate	1 vial	-20 °C
G-2	20X Rabbit Anti-phospho (Tyr992) EGFR Concentrate	1 vial	
G-3	20X Rabbit Anti-phospho (Tyr1068) EGFR Concentrate	1 vial	
G-4	20X Mouse Anti-phospho (Activated) EGFR Concentrate	1 vial	
H	80X Rabbit Anti-EGFR Concentrate	1 vial	
I-1	HRP Conjugated Anti-Rabbit IgG Concentrate	1 vial (10 µl)	
I-2	HRP Conjugated Anti-Mouse IgG Concentrate	1 vial (10 µl)	
J	TMB Substrate	2 vials (12 ml/ea)	2-8 °C
K	Stop Solution**	1 vial (14 ml)	

the 6 month expiration date. Avoid repeated freeze-thaw cycles.

*For up to 3 months (unless otherwise stated) or until expiration date.
**Contains 0.2 M Sulfuric Acid

III. ADDITIONAL MATERIALS REQUIRED

1. A model cell line, protein tyrosine kinase inhibitors, growth factors or cytokines
2. Microplate reader capable of measuring absorbance at 450 nm
3. 37 ^{o}C incubator
4. Precision pipettes to deliver 2 µl to 1 ml volumes
5. Adjustable 1-25 ml pipettes for reagent preparation
6. 100 ml and 1 liter graduated cylinders
7. Absorbent paper
8. Distilled or deionized water

9. Orbital shaker or oscillating rocker

IV. REAGENT PREPARATION

NOTE: *Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.*

NOTE: *Briefly centrifuge (~1,000g) ITEMS G, H, and I before opening to ensure maximum recovery.*

	ITEM	COMPONENT	PREPARATION	EXAMPLE
	A	Uncoated 96-Well Microplate	No Preparation	N/A
	B	20X Wash Buffer A Concentrate	Dilute 20-fold with distilled or deionized water	25 ml of concentrate + 475 ml of water = 500 ml of 1X working solution
	C	20X Wash Buffer B Concentrate		
	D	Fixing Solution	No Preparation	N/A
	E	30X Quenching Buffer Concentrate	Dilute 30-fold with 1X Wash Buffer	1 ml of concentrate + 29 ml of wash buffer = 30 ml of 1X working solution
	F	5X Blocking Buffer Concentrate	Dilute 5-fold with distilled or deionized water	20 ml of concentrate + 80 ml of water = 100 ml of 1X working solution
PRIMARY ANTIBODY	G-1	20X Mouse Anti-phospho (Tyr845) EGFR Concentrate	1. Reconstitute by adding 100 µl of 1X Blocking Buffer to each vial. 2. Pipette up and down to mix. 3. Dilute reconstituted stock 20-fold with 1X Blocking Buffer	10 µl of reconstituted stock + 190 µl of 1X Blocking Buffer = 200 µl of 1X working solution
	G-2	20X Rabbit Anti-phospho (Tyr992) EGFR Concentrate		
	G-3	20X Rabbit Anti-phospho (Tyr1068) EGFR Concentrate		
	G-4	20X Mouse Anti-phospho (Activated) EGFR Concentrate		
	H	80X Rabbit Anti-EGFR Concentrate	1. Reconstitute by adding 100 µl of 1X Blocking Buffer to each vial . 2. Pipette up and down to mix. 3. Dilute reconstituted stock 80-fold with 1X Blocking Buffer	10 µl of reconstituted stock + 790 µl of 1X Blocking Buffer = 800 µl of 1X working solution
SECONDARY ANTIBODY	I-1	HRP Conjugated Anti-Rabbit IgG Concentrate	Dilute 2000-fold with 1X Blocking Buffer for Item G3 and Item H	2 µl of concentrate + 3998 µl of 1X Blocking Buffer = 4 ml of 1X working solution
			Dilute 5000-fold with 1X Blocking Buffer for Item G2	2 µl of concentrate + 9998 µl of 1X Blocking Buffer = 10 ml of 1X working solution
	I-2	HRP Conjugated Anti-Mouse IgG Concentrate	Dilute 2000-fold with 1X Blocking Buffer for Item G1	2 µl of concentrate + 3998 µl of 1X Blocking Buffer = 4 ml of 1X working solution
			Dilute 1000-fold with 1X Blocking Buffer for Item G4	2 µl of concentrate + 1998 µl of 1X Blocking Buffer = 2 ml of 1X working solution
	J	TMB Substrate	No preparation	N/A
	K	Stop Solution		

V. ASSAY PROCEDURE:

NOTE: *ALL incubations and wash steps must be performed under gentle rocking or rotation (~1-2 cycles/sec).*

1. Design your experiment. For example, see Figure 2 below.

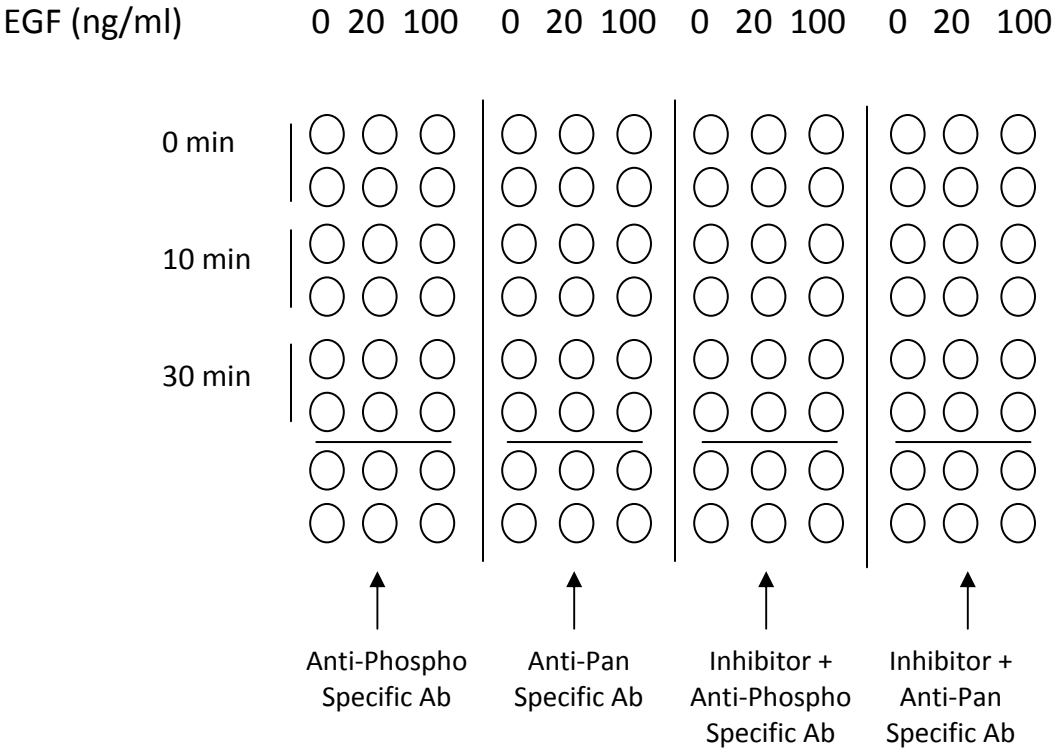

Fig. 2. Example of plate layout for RayBio® cell-based assay

OPTIONAL: *If seeding HUVECs, HMEC-1 or other loosely attached cells, coat the Uncoated 96-Well Microplate (ITEM A) by adding 100 µl poly-L-Lysine (Recommended Sigma Aldrich, Cat#: P4832) into each well and then follow manufacturer's instructions. A pre-coated CellBIND® microplate or other poly-lysine treated tissue culture plate may be used in place of ITEM A.*

2. Seed 100 µl of 10,000 to 30,000 cells into each well of the Uncoated 96-Well Microplate (ITEM A) provided and incubate overnight at 37°C with 5% CO_2.

NOTE: *The optimal cell number used will vary on the cell line and the relative amount of protein phosphorylation. More or less cells may be used but this must be determined empirically.*

NOTE: *The cells can be starved ~4-24 hours (depending on cell line) prior to treatment with inhibitors or activators.*

3. Apply various treatments, inhibitors (such as siRNA or chemicals) or activators according to manufacturer's instructions and incubate for the desired time points.

NOTE: *It is recommended to dissolve inhibitors or activators into serum-free cell culture medium before treating the cells (unless otherwise stated in the manufacturer's instructions.)*

4. Discard the cell culture medium by flipping the microplate upside down and **gently** tapping the bottom of the microplate over a sink.

5. Wash by pipetting 200 µl of the **prepared 1X** Wash Buffer A (ITEM B) into each well. Discard the wash buffer (same as step 4) and wash 2 more times for a total of 3 washes using fresh wash buffer each time. After the final wash, gently blot the microplate onto a paper towel to remove any excess/remaining buffer.

NOTE: *To avoid cell loss, do not pipette directly onto the cells. Instead, gently dispense the liquid down the wall of cell culture wells. Also avoid the use of vacuum suction or too forcefully tapping the microplate when discarding any solution.*

6. Add 100 µl of Fixing Solution (ITEM D) into each well and incubate for 20 minutes at room temperature.

NOTE: *The fixing solution is used to permeabilize the cells.*

7. Repeat wash step 5.

8. Add 200 μl of **prepared 1X** Quenching Buffer (ITEM E) into each well and incubate 20 minutes at room temperature.

NOTE: *The quenching buffer is used to minimize the background response.*

9. Wash **4** times with 1X Wash Buffer A.

10. Add 200 μl of the **prepared 1X** Blocking Buffer (ITEM F) into each well and incubate for 1 hour at 37^{o}C.

11. Wash **3** times with the **prepared 1X** Wash Buffer **B** (ITEM C).

NOTE: *If needed, the microplate may be stored at -80^{o}C for several days after this wash.*

12. Add 50 μl of the **prepared 1X** primary antibody (ITEM G1, G2, G3, G4, or H) into each corresponding well and incubate for 2 hours at room temperature.

13. Wash **4** times with 1X Wash Buffer B.

14. Add 50 μl of the **prepared 1X** HRP Conjugated secondary antibody (ITEM I-1 or I-2) into each well and incubate for 1 hour at room temperature.

NOTE: *Item I-1 is the secondary antibody for Items G2, G3, and H (primary antibody). Item I-2 is the secondary antibody for Items G1 and G4 (primary antibody).*

15. Repeat step 13.

16. Add 100 μl of the TMB Substrate (ITEM J) into each well and incubate for 30 minutes at room temperature **in the dark**.

17. Add 50 μl of the Stop Solution (ITEM K) into each well. Read at 450 nm immediately.

VI. ASSAY PROCEDURE SUMMARY

1. Seed 10,000 to 30,000 cells into each well and incubate overnight.

⇩

2. Apply various treatment, inhibitors or activators according to manufacturer's instructions.

⇩

3. Add 100 µl of Fixing Solution into each well and incubate for 20 minutes at room temperature.

⇩

4. Add 200 µl of prepared 1X Quenching Buffer and incubate for 20 minutes at room temperature.

⇩

5. Add 200 µl of prepared 1X Blocking Buffer and incubate for 1 hour at 37^{o}C.

⇩

6. Add 50 µl of prepared 1X primary antibody into each well and incubate for 2 hours at room temperature.

⇩

7. Add 50 µl of prepared 1X HRP Conjugated Secondary Antibody and incubate for 1 hour at room temperature.

⇩

8. Add 100 µl TMB Substrate and incubate 30 minutes at room temperature.

⇩

9. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

VII. QUALITY CONTROL DATA

Representative results of Cell-Based EGFR (Tyr992) are shown below:

1. Seeded 20,000 A431 cells into appropriate wells of the microplate. Cells were incubated at 37°C in 5% CO_2 overnight.

2. Added 50 µl of different concentrations of stimulators (rhEGF concentration for A431 cells: 0, 20 or 100 ng/ml in serum free DMEM) to appropriate wells (shown below). Then incubated for 10 min at 37°C.

3. Discarded the solution and wash 3 times with 1X Wash Buffer A (200 µl each) immediately. Then tapped the plate upside down to remove all of excess wash buffer. The protocol was then followed as stated.

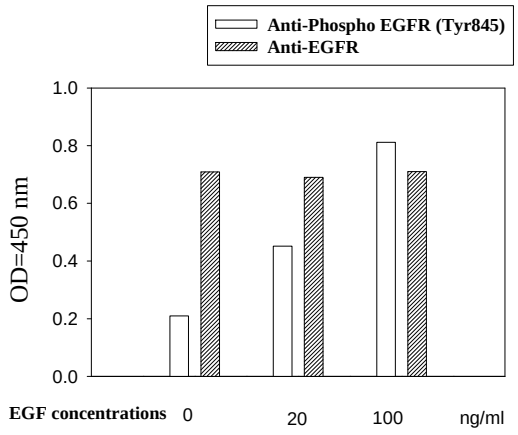

Fig. 3-1. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

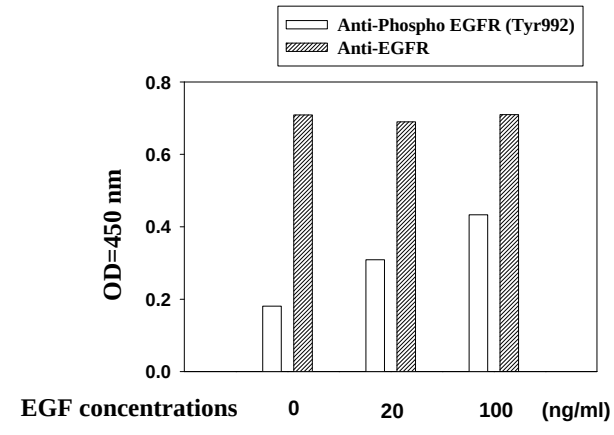

Fig. 3-2. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

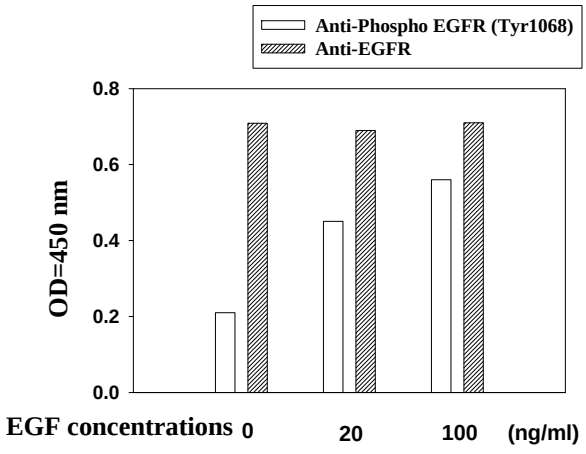

Fig. 3-3. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

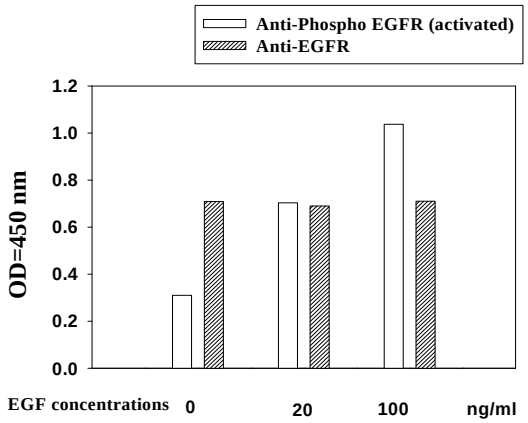

Fig. 3-4. A431 cells were stimulated by different concentrations of EGF for 20 min at 37°C

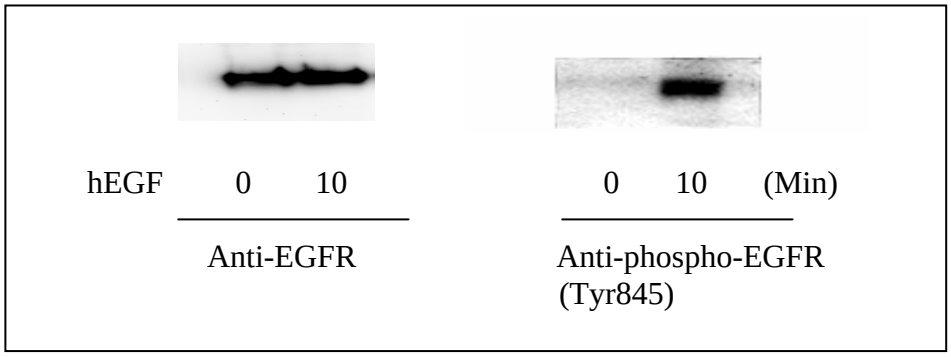

Fig. 4-1. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells. Phospho-EGFR (Tyr845) and EGFR antibodies were used in both detection assays.

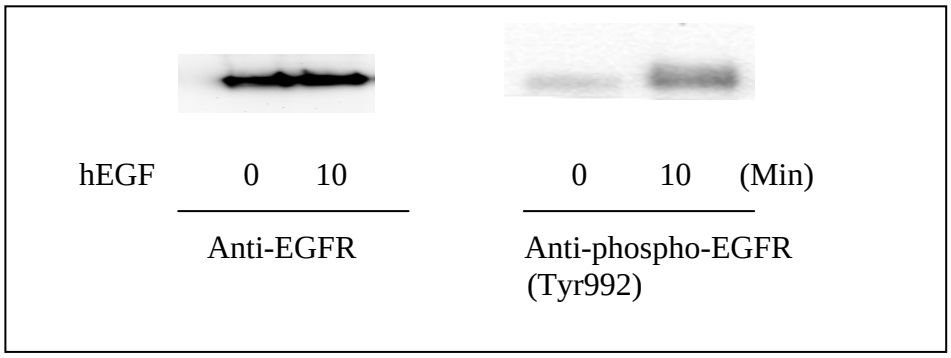

Fig. 4-2. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells. Phospho-EGFR (Tyr992) and EGFR antibodies were used in both detection assays

Fig. 4-3. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells. Phospho-EGFR (Tyr1068) and EGFR antibodies were used in both detection assays.

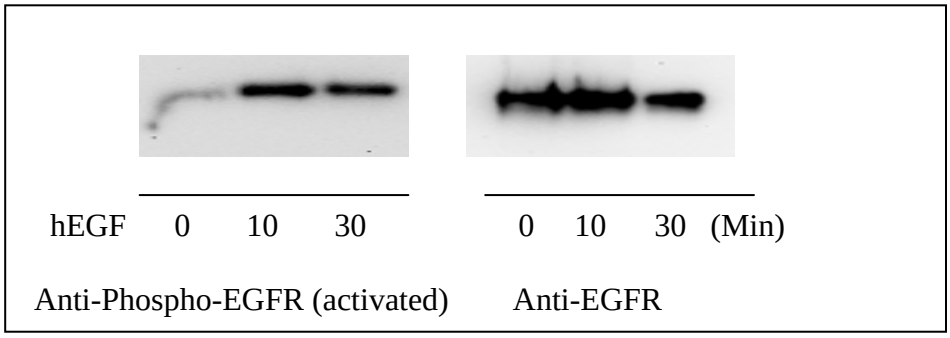

Fig. 4-4. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells. Phospho-EGFR (activated) and EGFR antibodies were used in both detection assays.

VIII. REFERENCES:

1. Michael J. Clemens and Michael C. 1997. *Protein Phosphorylation in Cell Growth Regulation*. 1 Edition.
2. Fu, X.Y., et al. 1993 *Cell* 74:1135.
3. Darnell, J.E. 1997 *Science* 277:1630.
4. Kanai, M., et al. 2003. *Oncogene* 22:548-554.
5. Smith, P.D. & Crompton, M.R. 1998. *Biochem. J.* 331:381.

IX: TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1. Improper storage of the ELISA kit	1. Store the kit according to manual instructions. Keep substrate solution in dark.
	2. Improper dilution	2. Ensure correct preparation of antibody and reagents.
	3. Cells drop off from the wells	3. Some of treatments may make cells drop off the wells. Reduce inhibitor or activator concentration.
2. High background	1. Inadequate washing	1. Be sure to remove all of washing solution and follow the recommendation for washing.
	2. Too much cells	2. Reduce the cell number.
3. Large CV	1. Inaccurate pipetting	1. Check pipette.
	2. Remaining wash buffer in the well	2. Remove all of wash buffer.
	3. Cells drop off from the wells	3. Please don't directly face the cells with tips when adding reagents or wash buffer.

NOTES:

Note:

This product is for research use only.

©2004 RayBiotech, Inc.